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FGFR4 and β-Klotho in Metastatic Prostate Cancer

by

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ABSTRACT

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Fibroblast growth factors and fibroblast growth factor receptors have been associated with the aggressiveness and progression of Prostate Cancer (PCa). Also, β-Klotho is a known co-receptor with FGFR4 for FGF19 in the liver however, the role of this co-receptor pair remains unclear in the setting of PCa.

I demonstrated that FGFR4 and KLB mRNA and protein are highly expressed in PCa cells when compared to bone marrow stromal cells, a common site of metastasis. I also provide support for the association of FGFR4 and KLB in PCa, suggesting a functional co-receptor pair capable of altering cellular signaling. FGFR4-KLb may also provide some level of protection to PCa cells from chemotherapeutics.

This analysis of FGFR4 and KLB expression and signaling in PCa has provided novel insights into phenotypic alterations during PCa progression while also providing new avenues of study to further explore the role and importance of this exciting co-receptor complex.

ACKNOWLEDGEMENTS

Many people contributed greatly to my success at Rice. I would like to thank:

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ABBREVIATIONS

ACC2	Acetyl-CoA carboxylase 2
BMPs	Bone Morphogenic Proteins
BMSCs	Bone Marrow Stromal Cells
BSA	Bovine Serum Albumin
CT	Comparative Threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
FGFs	Fibroblast Growth Factors
FGFRs	Fibroblast Growth Factor Receptors
HSPGs	Heparan Sulfate Proteglycans
HRP	Horseradish Peroxidase
KLB	β-Klotho
NSE	Neuron Specific Enolase
OC	Osteocalcin
OPN	Osteopontin
PCa	Prostate Cancer

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PLA	Proximity Ligation Assay
PMSF	Phenylmethanesulfonyl fluoride
PS	Penicillin-Streptomycin
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBST	Tris-buffered saline and Tween 20

Chapter 1

Introduction

1.1. Metastatic prostate cancer

Prostate Cancer (PCa) is the most commonly diagnosed and second leading cause of cancer deaths in American men [9]. PCa originates as an epithelial disease, yet in metastatic disease, these cells primarily “home” to mesenchymal tissues such as bone [12, 31]. The bone microenvironment is quite hostile to epithelial cells and inherently prevents survival of epithelial cancer cells. However, PCa metastatic cells have been shown to adopt osteoblast characteristics by upregulating osteopontin (OPN), osteocalcin (OC), and bone morphogenic proteins (BMPs) in order to increase survival in the hostile bone microenvironment [3, 12], a process that has been termed “osteomimicry” [12]. Many of the phenotypic adaptations that enable PCa cells to survive in hostile tissues can be driven through mechanisms such as osteomimicry as well as epithelial-mesenchymal transition (EMT). The process of EMT is thought to be a major mechanism that allows metastasis to leave the primary tumor and thrive in distal tissues like bone, yet many of the molecular mechanisms of this process remain unclear [3,12, 31]. EMT is

initiated upon the loss of E-cadherin and other epithelial adhesion and extracellular matrix (ECM) proteins, allowing PCa cells to move from their tissue of origin. In order to survive in distal tissues, PCa cells are thought to adopt a mesenchymal phenotype by upregulating mesenchymal association proteins such as N-cadherin [31]. In Figure 1.1, PCa progression is depicted along with the corresponding cells of the LNCaP progression model of human PCa that were studied in this project. During each stage of PCa progression, cancer cells become more phenotypically mesenchymal and osteoblastic, thus increasing the potential for tumor formation and metastasis of these cells. Additionally, during EMT metastatic PCa cells often alter growth factor signaling to 'mirror' that of bone cells and these alterations maximize survivability and proliferation in the bone microenvironment [7, 12]. However, the complete picture of PCa adaptations of growth factor expression and signaling in the bone microenvironment remain unclear. A more complete understanding of the phenotypic alterations of PCa cells will help identify select pathways upon which cancer cells rely to survive in otherwise hostile environments and potentially direct effective and novel therapies.

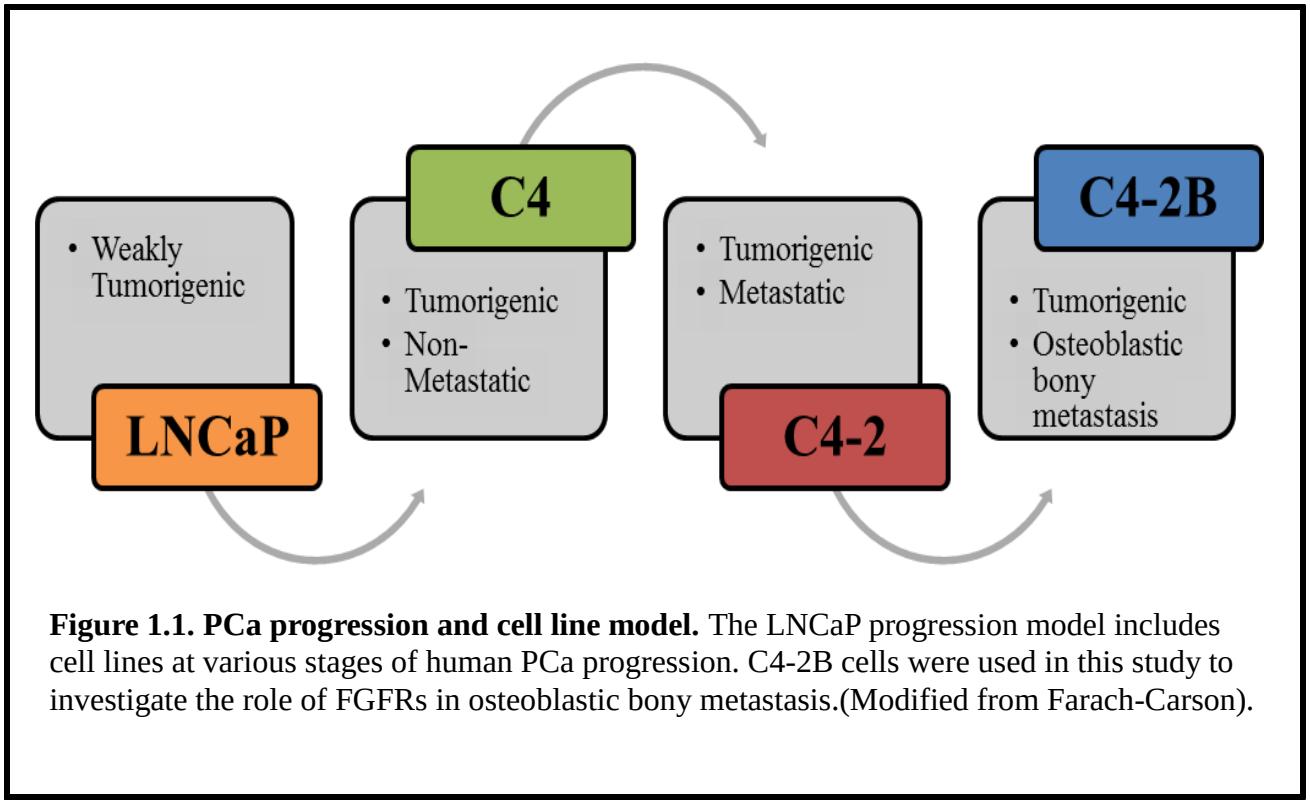


Figure 1.1. PCa progression and cell line model. The LNCaP progression model includes cell lines at various stages of human PCa progression. C4-2B cells were used in this study to investigate the role of FGFRs in osteoblastic bony metastasis.(Modified from Farach-Carson).

1.1.1. Fibroblast growth factors and fibroblast growth factor receptors in prostate cancer

In clinical disease, overexpression of FGFs and FGFRs has been associated with the initiation, progression, and aggressiveness of PCa [24, 30]. The FGFR family is comprised of four distinct receptors, FGFR-1 to FGFR-4, sharing between 55% and 72amin acid homology [28]. FGFRs are single-pass transmembrane proteins with three extracellular Ig-like domains (IgI-III) and stretches of acidic residues interspersing each of these domains [1]. Ig-like domains, particularly IgIII, are the sites to which FGFs bind and subsequently activate FGFR signaling. To provide ligand-binding diversity, FGFRs 1-3 undergo alternate splicing of the IgIII domain where FGFs primarily bind [1, 28]. Interestingly, FGFR4 is only found as one isoform and does not undergo alternate splicing in the IgIII domain and is also shown to be the FGFR most closely associated with PCa progression and aggressiveness [5, 23, 24, 28, 30]. Previously published work from the Farach-Carson laboratory has shown FGFR4 mRNA expression in C4-2B PCa cells, but little to none in HS27a bone marrow stromal cells [23]. FGFRs also contain a positively charged heparin/heparan sulfate binding domain that is part of the IgII domain and is required for binding canonical FGFs and activation of FGFR signaling [5, 32]. In the most-recently proposed oligomerization model of HSPG-mediated FGFR signaling, two FGFRs and two FGFs dimerize and are stabilized further upon HSPG binding, forming a ternary complex (Figure 1.2) [28, 32].

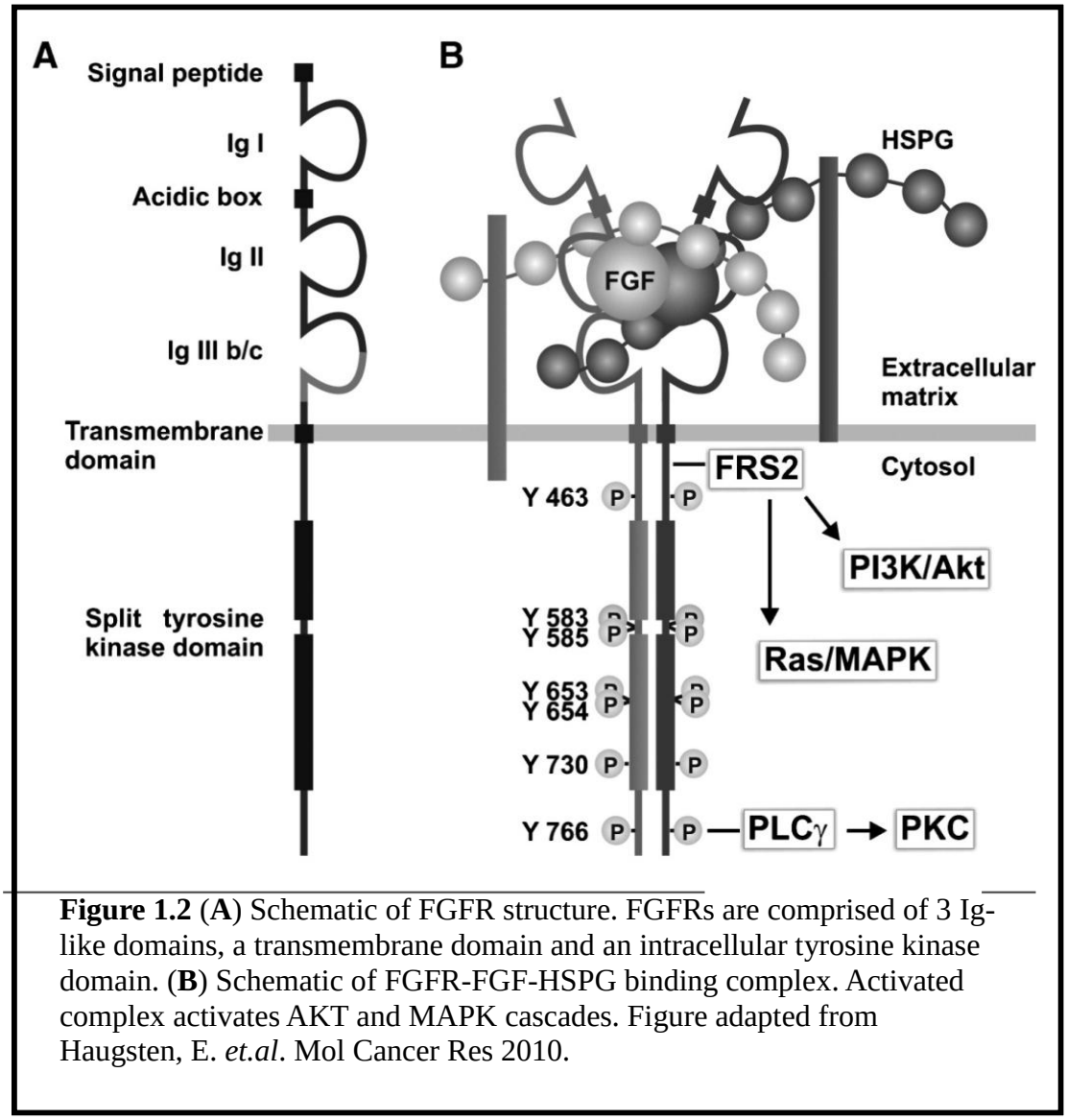


Figure 1.2 (**A**) Schematic of FGFR structure. FGFRs are comprised of 3 Ig-like domains, a transmembrane domain and an intracellular tyrosine kinase domain. (**B**) Schematic of FGFR-FGF-HSPG binding complex. Activated complex activates AKT and MAPK cascades. Figure adapted from Haugsten, E. *et.al*. Mol Cancer Res 2010.

1.1.2. FGFR4 in prostate cancer

Each FGFR is activated by a subset of distinct FGFs, and FGFR4 is primarily activated by FGF1, FGF8, FGF9, and exclusively by the FGF19 subfamily [1, 16]. Once the FGF-FGFR-HSPG complex is formed, the cytosolic RTK domain, containing up to seven tyrosine residues, transphosphorylates and subsequently activates various signaling cascades. Phosphorylated FGFRs activate the Ras/MAPK and the PI3K/AKT pathways, both of which have been strongly associated with mitogenesis and other cell survival pathways in cancer [16, 21]. However, FGFR4 has been shown to activate the Ras/MAPK, PLCγ, and ATK pathways to a lesser extent than the other FGFRs even though FGFR4 binds mitogenic FGFs [29, 30, 35]. In their study, Vainikkak *et al*. determined that compared to FGFR1, FGFR4 only weakly induced MAPK phosphorylation, tyrosyl phosphorylation of PLCγ, and was not found to bind GRB2 even though an increase in mitogenesis was seen [33]. Wang *et al*. also found that compared to FGFR1, FGFR4 induced a lower degree of ERK phosphorylation and lower RNA expression of two potent transcription factors *fos* and *tis11* [35]. These data suggest that activated FGFR4 does not induce mitogenic signaling pathways as strongly as FGFR1-3 and may be activating other nonmitogenic pathways that also provide increased survival in the hostile bone environment. FGFR4 has also been implicated in the regulation of cellular metabolism. Interestingly, activation of FGFR4 by FGF19 reduced expression of a potent metabolic regulator acetyl-CoA carboxylase2 (ACC2), suggesting oxidation of fatty acids and increased cellular metabolism [6]. By exploiting FGFR4 and FGF19 signaling that increase cellular metabolism, PCa cells may gain a competitive advantage

over the surrounding microenvironment, thus increasing tumor growth and survival in the surrounding bone marrow microenvironment.

1.1.3. FGFR4 and β-Klotho in prostate cancer

As discussed previously, FGF 1, 8, and 9 are activators of FGFR4 and act as potent mitogens that have been implicated in PCa progression. FGFR4 is also the exclusive receptor for FGF19s [1, 16, 27]. FGF19s are potent regulators of metabolism and vitamin D regulation of minerals in bone and unlike the canonical FGFs, the FGF19 subfamily has weak binding affinity for HSPGs allowing FGF19s to evade much of the extracellular matrix and act as endocrine signaling factors [1, 21]. However, the reduced heparin/heparan sulfate-binding affinity also reduces the ability of FGF19s to bind FGFRs. In order for FGF19 and FGFR4 interaction, the transmembrane cofactor β-Klotho (KLB) is required [15]. The Klotho gene family, composed of α-Klotho and KLB are transmembrane glycoproteins whose extracellular domain consists of beta-glycosidase-like repeats and share approximately 41% amino acid homology [14, 21]. KLB is primarily expressed in the liver and to lesser extent in the prostate, pancreas, kidneys and adipose tissues and is known to enhance FGF19 association with FGFR4 in humans to regulate lipid and glucose metabolism. Interestingly, the FGFR4-KLB receptor complex has been shown to "induce apoptosis and inhibit tumor cell proliferation" in the liver [21]. Together, these studies suggest that FGFR4 and KLB are involved in regulating both mitogenic and metabolic pathways to regulate cancer cell behavior; however, few studies have investigated the signaling pathways of FGFR4 and KLB in the setting of PCa bone metastasis. The experiments that I undertook were designed to elucidate the expression levels of FGFR4 and KLB in both PCa and bone marrow stromal

cells (BMSCs) as well as to determine the role of these co-receptors in mitogenic, cell survival and/or metabolic signaling thus provide attractive signaling targets for future cancer treatments. If FGFR4 and KLB are more highly expressed in PCa, FGFR4 and KLB may also provide enhanced mitogenic, survival and/or metabolic signaling that enables PCa cell survival and growth in an otherwise hostile bone microenvironment. An understanding of FGFR4 and KLB expression and signaling will not only increase our knowledge of tumor biology, but FGFR4 and KLB may be an attractive coreceptor complex for targeting anti-cancer therapies.

Chapter 2

Materials and Methods

2.1. Cell Culture

LNCaP, C4, C4-2 and C4-2B cells (a gift of Dr. Leland Chung) were grown in T-medium (Gibco BRL/Life Technologies, Carlsbad, CA) and supplemented with 10% (v/v) heat-inactivated fetal bovine (FBS), 1%(v/v) penicillin/streptomycin and 1%(v/v) L-glutamine. Growth media was changed every 48 hours and cells were passaged at 80% confluency with 0.25% (w/v) trypsin containing 1X ethylenediamine tetraacetic acid (EDTA). HS27a and HepG2 (ATCC, Manassas, VA) cells were grown in Low Glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) FBS and 1% Penicillin-Streptomycin (PS). Medium was changed every 48 hours and passaged at 80% confluency with 0.25% (w/v)trypsin containing 1X EDTA.

2.1.1. FGF19 Iand FGF1 treatments

C4-2B cells were grown in 6-well plates and maintained as described above. At 50% confluency (judged by microscopic inspection), cells were placed into fresh media for 48 h prior to all FGF treatments. Cells were then incubated with 0.1% (w/v) BSA vehicle control, 200 ng/mL recombinant human FGF19 (R&D Systems, Minneapolis, MN) or 200 ng/mL of recombinant human FGF1 (R&D Systems, Minneapolis, MN) for 6 h or 24 h in a humidified atmosphere of air:CO_2 (95:5; v/v). Protein then was extracted for western blot analysis. Protein was extracted from a 6-well plate using RIPA lysis buffer, 1% (v/v) phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail including inhibitors of serine proteases, amino-peptidases, cysteine proteases, and metalloproteases and 1% (v/v) sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined using a BCA assay (Thermo Fisher Scientific). Proteins were separated using NUPAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) for 55 minutes at 200 V. Proteins were transferred to a nitrocellulose membrane at 4°C for 2 h at 60 V. The membrane was blocked in a solution of 3% (w/v) BSA in TBST for 2 h at 25°C while shaking. The membranes then were incubated with either 5µg/mL FGFR4 antibody(sc-136988, Santa Cruz Biotechnology) and 5µg/mL, β-Klotho (LS-B3568, LifeSpan Biosciences, Seattle, WA) at 5µg/mL, p-ERK antibody (05-797R, EMD Millipore)at a 1:2,000 dilution, p-S6 antibody (4858, Cell Signaling Technology, Inc) at a 1:2,000 dilution, or β-actin antibody (Ab8226, Abcam) at a 1:10,000 dilution and incubated overnight at 4°C while shaking. The blots were then washed three times with Tris-

buffered saline with tween 20 (TBST) for 5 minutes each with gentle rotary agitation. Blots then were incubated with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies at 1 1:500 dilution (Sigma-Aldrich, St. Louis, MO). Again, the blot was washed three times with TBST and finally incubated with Pierce ECL Western Blotting Substrate (Invitrogen) for 10 minutes at 25°C. Prior to being re-probed with other primary antibodies, blots were incubated with Restore Western Blot Stripping Buffer (Pierce) according to the manufacturer's protocol.

2.1.2. Camptothecin treatments and live/dead assay

C4-2B cells were grown in 6-well plates and maintained as described above. At 50% confluency, cells were placed in fresh media for 48 h prior to all FGF treatments. Cells were then incubated with 0.1% BSA vehicle control, 200ng/mL FGF19 or 200ng/mL FGF1 for 6 h at 37°C in a humidified atmosphere of air:CO_2 (95:5, v/v). Media was removed and cells were then incubated with 1 μL of vehicle control DMSO or 0.574μM camptothecin(MP Biomedicals, Solon, OH) for 24 h. following FGF and drug treatments, Live/dead staining was carried out as follows: Growth medium was removed and Calcein AM and Ethidium homodimer nucleic acid stain (Invitrogen) were incubated with cells at 1:1000 (v/v) in Dulbecco's Phosphate-Buffered Saline (DPBS). 1mL of live/dead mixture was added for 15 min then cell fluorescence was visualized a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.2. RNA isolation and qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following manufacturer's protocol. cDNA was synthesized with a qScript cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, MD) according to manufacturer's protocol. Equal volumes of cDNA were used as template for qRT-PCR using iQ SYBR Green Supermix (Bio-Rad) according to manufacturer's protocol (See table 2 for all primer sequences used).

Target	Primer Sequence
β-Klotho FWD	5'-ACGGCGACATGGACATTTAC-3'
β-Klotho REV	5'-CATCCTCCAGAGCCTGGTC-3'
FGFR4 FWD	5'-AGCCAGGTGAGGAGGAGCCA-3'
FGFR4 REV	5'-GGCCCAGGCACACTCAGCAG-3'
ACC2 FWD	5'-TTATCTGACCACAGGTGAAGCTGAGA-3'
ACC2 REV	5'-GCTCCGGAAGTTTAGGGTTTTCTAAAG-3'

Table 2.1-Sequences of oligonucleotides used in this study

2.3. Western blot analysis

C4-2B cells were grown as described above. Protein was extracted from a 6-well plate using RIPA lysis buffer, 1% (v/v) phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) protease inhibitor cocktail including inhibitors of serine proteases, amino-peptidases, cysteine proteases, and metalloproteases, and 1% (w/v) sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined using a

BCA assay (Thermo Fisher Scientific). Proteins were separated using NUPAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) for 55 minutes at 200 V. Proteins were transferred to a nitrocellulose membrane at 4°C for 2 h at 60 V. The membrane was blocked in a solution of 3% (w/v) BSA in TBST for 2 h at 25°C with gentle rotary agitation. The membranes then were incubated with either 5μg/mL FGFR4 antibody(sc-136988, Santa Cruz Biotechnology) and 5μg/mL, β-Klotho antibody (LS-B3568, LifeSpan Biosciences, Seattle, WA) at 5μg/mL, p-ERK antibody (05-797R, EMD Millipore)at a 1:2,000 dilution, p-S6 antibody (4858, Cell Signaling Technology, Inc) at a 1:2,000 dilution, or β-actin antibody (Ab8226, Abcam) at a 1:10,000 dilution and incubated overnight at 4°C with gentle rotary agitation. The blots were then washed three times with TBST for 5 minutes each with gentle rotary agitation. Blots then were incubated with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Again, the blot was washed three times with TBST and incubated with Pierce ECL Western Blotting Substrate (Invitrogen). Prior to being re-probed with other primary antibodies, blots were incubated with Restore Western Blot Stripping Buffer (Pierce) according to the manufacturer's protocol.

Target	Host	Company	Catalog #
FGFR4	Mouse monoclonal	Santa Cruz Biotechnology	sc-136988
β-Klotho	Rabbit polyclonal	LifeSpan BioSciences	LS-B3568
p-ERK	Rabbit monoclonal	EMD Millipore	05-797R
p-S6	Rabbit monoclonal	Cell Signaling Technology	4858
β-actin	Mouse monoclonal	Abcam	Ab8226

Table 2.2-- List of antibodies used in this study

2.4. Immunofluorescence

Cells were grown on square coverslips (VWR International, Randor, PA) in 6-well plates as described above. Medium was removed and cells were fixed in 4% (w/v) paraformaldehyde (PFA) (MP Biochemicals, Solon, OH) in ddH_2O. PFA was removed and cells were washed in phosphate buffered saline (PBS). Cells were then blocked in 1% (w/v) BSA in PBS for 30 minutes at 25°C in a humidified chamber. Cells were subsequently incubated with 5μg/mL mouse anti-FGFR4 (Abcam, Cambridge, MA) and 5μg/mL rabbit anti-β-Klotho (LifeSpan Biosciences, Seattle, WA) at 4°C overnight. Cells were washed in PBS followed by 1:500 solutions of anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 568 (Invitrogen) for one hour at 25°C, washed in PBS, incubated for 1 minute in a 1μg/mL 4',6-diamidino-2-phenylindole (DAPI) solution (EMB Millipore) and washed in PBS before mounting the coverslips on slides. Slides were imaged with a Zeiss

Axioplan 2 microscope at 40X magnification (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.5. *in situ* Proximity Ligation Assay

C4-2B and HS27a cells were grown on coverslips in 6-well plates as described above. Medium was removed and cells were fixed in 4% (w/v) paraformaldehyde (PFA) (MP Biochemicals, Solon, OH) in ddH_2O. PFA was removed and cells were washed in phosphate buffered saline (PBS). Cells were then blocked in 1% (w/v) BSA in PBS for 30 minutes at 25°C. Cells were subsequently incubated with 5µg/mL mouse anti-FGFR4 (Abcam, Cambridge, MA) and 7µg/mL rabbit anti-β-Klotho (LifeSpan Biosciences, Seattle, WA) at 4°C overnight. As a control for non-specific PLA signal, mouse anti-Neuron-Specific Enolase (NSE) was used in place of anti-FGFR4 antibody. Following washes with PBS, the *in situ* Proximity Ligation Assay was performed according to the manufacturer's protocol using Duolink II PLA probe anti-Mouse PLUS and Duolink II PLA probe anti-Mouse MINUS and detected with Duolink II Detection Reagents Red (OLink Bioscience, Uppsala, Sweden). Coverslips were mounted with DAPI mounting media (OLink) on microscope slides and images were obtained with a Zeiss Axioplan 2 microscope at 40X magnification (Carl Zeiss Microscopy, Thornwood, NY, USA). All FGFR4-KLB PLA images and minus KLB primary antibody control samples were color-enhanced in parallel to reduce background. The mean PLA signal from the NSE-KLB control experiment was subtracted from the mean FGFR4-KLB PLA for final statistical analysis.

2.6. β-Klotho knockdown with siRNA

C4-2B cells were plated and maintained in 12-well plates as described above. At 60% confluency, media was removed and 40nM KLB siRNA (Origene, Rockville, MD) was added in serum-free Dulbecco's Modified Eagle Medium (Invitrogen) for 24 h. siRNA containing media was removed and the cells were subsequently treated with 200ng/mL FGF19 or FGF1 in appropriate growth media for 24 h. Protein was collected and western blotting analyses were conducted as described above.

2.7. Statistical analysis

To determine significance of FGFR4 expression levels between PCa and BMSCs, a One-way ANOVA test and a secondary Tukey test for multiple comparisons using GraphPad In-stat software. For PLA analysis, a Welch's corrected t test was used using GraphPad In-stat software.

Chapter 3

FGFR4 and β-Klotho expression and co-localization during prostate cancer progression

3.1. Characterization of FGFR4 and β-Klotho expression in the LNCaP progression model of prostate cancer

3.1.1. Quantitative RT-PCR of FGFR4

Previous published studies from our laboratory have shown that FGFR4 mRNA is expressed in C4-2B, but little or none is detectible in HS27a bone marrow stromal cells [23]. To quantitatively compare mRNA expression of FGFR4 between these PCa cells and bone stromal cells, I conducted qRT-PCR experiments in LNCaP, C4, C4-2, C4-2B and HS27a cells. FGFR4 primers were selected based on sequence specificity by NCBI Blast and designed to span introns in order to avoid genomic DNA amplification. For

each cell type, samples were analyzed in biological triplicate and the mean comparative threshold (CT) values ± standard deviation (SD) was normalized to β-actin. In Figure 3.1, C4-2 and C4-2B cells expressed the highest levels of FGFR4. when compared to HS27a cells. C4, and HS27a cells express approximately 4.0 and 2.5 fold less FGFR4 respectively when compared to C4-2B cells. These data are consistent with previous literature suggesting higher FGFR4 expression is associated with both agressivness and the metastatic potential of the PCa cells.

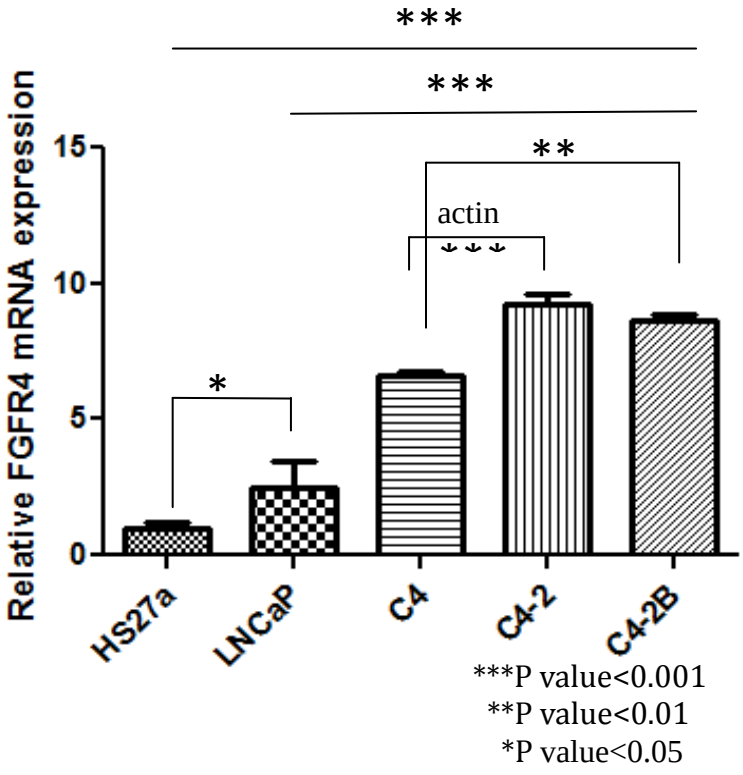


Figure 3.1. FGFR4 mRNA expression in PCa and BMSCs. All PCa cell lines have significantly more FGFR4 mRNA than HS27a cells. Relative FGFR4 mRNA expression in LNCaP, C4, C4-2 and C4-2B cells are 2, 6, 9 and 8-fold higher than HS27a cells, respectively.

3.1.2. qRT-PCR of β-Klotho

To determine KLB mRNA expression in PCa and bone stromal cells, I conducted similar RT-PCR experiments designed to determine FGFR4 mRNA expression in C4-2B and HS27a cells [23]. In Figure 3.2., a strong signal for KLB cDNA of approximately 281bp was found to be present in C4-2B cells, but not HS27a cells.

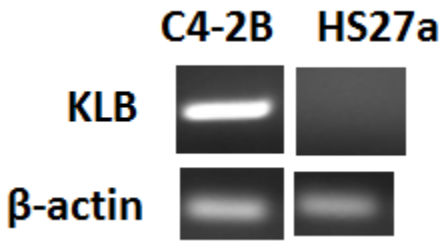


Figure 3.2. RT-PCR analysis of KLB mRNA. KLB mRNA is highly expressed in C4-2B cells but not HS27a cells

To more fully determine the extent of KLB expression in PCa cells, I conducted qRT-PCR experiments in the LNCaP series and HS27a cells. Primers were selected from a literature article exploring KLB expression in colonic tissues and similarly to the FGFR4 qRT-PCR experiments discussed above, each cell type was analyzed in biological triplicate with the mean comparative threshold (CT) values $\pm$SD was normalized to β-actin [47]. In Figure 3.3, KLB mRNA is expressed approximately 15-fold higher in LNCaP, 25-fold higher in C4, 26-fold higher in C4-2 and 33-fold higher in C4-2B when compared to HS27a cells (Figure 3.3). These data suggest that KLB is highly expressed in PCa cells when compared to BMSCs, similar to FGFR4 and these two proteins have the potential to form a functional co-receptor complex for enhanced PCa cell signaling in the bone marrow microenvironment.

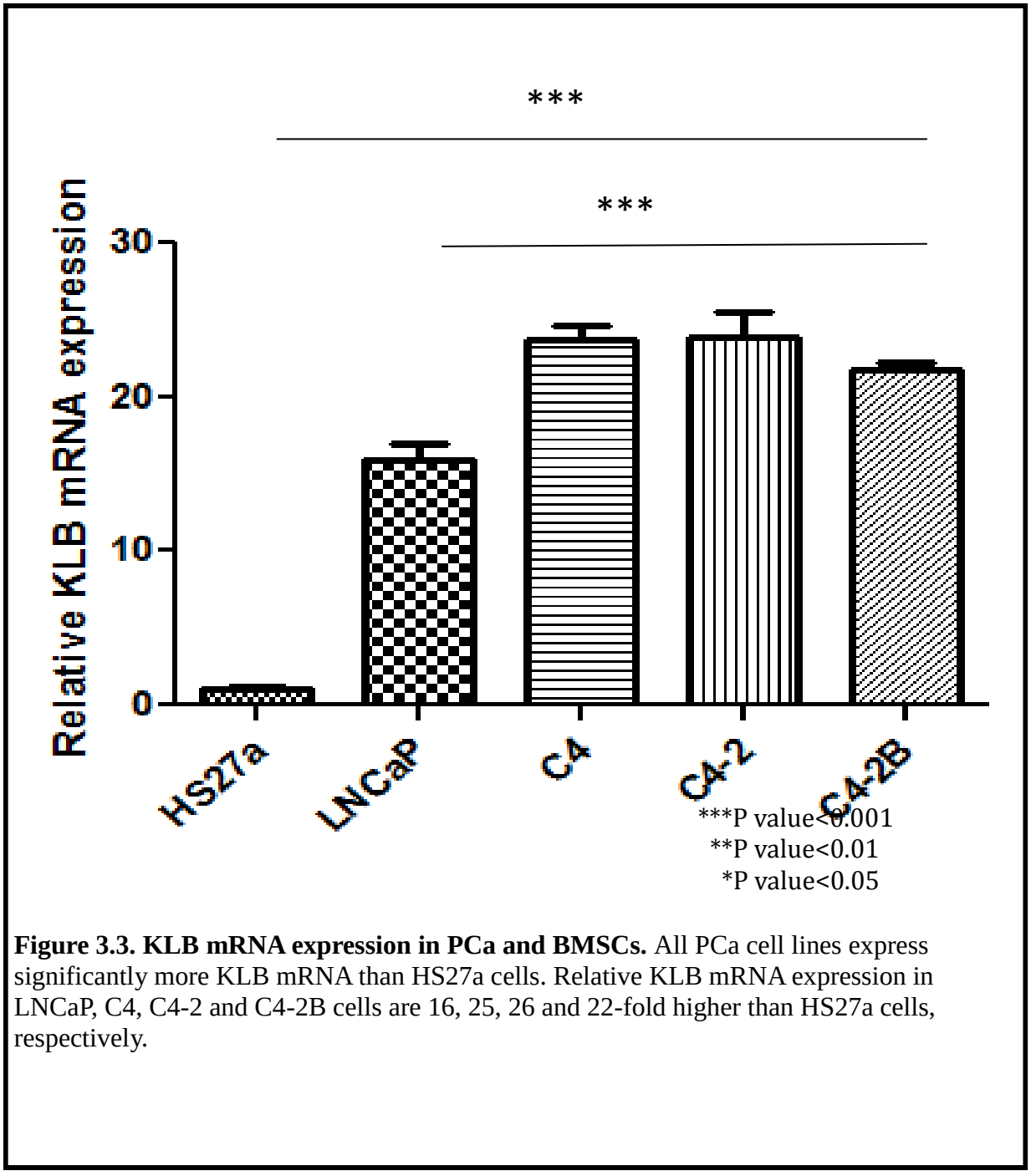


Figure 3.3. KLB mRNA expression in PCa and BMSCs. All PCa cell lines express significantly more KLB mRNA than HS27a cells. Relative KLB mRNA expression in LNCaP, C4, C4-2 and C4-2B cells are 16, 25, 26 and 22-fold higher than HS27a cells, respectively.

3.1.3. Western blot analysis of FGFR4 and β-Klotho in PCa and bone marrow stromal cells.

qRT-PCR demonstrates differences in mRNA levels of FGFR4 and KLB among the cell lines examined; however, it is also important to assay protein expression, especially when investigating the role of two cell surface receptors and their potential role in various cellular signaling pathways. To this end, western blotting was chosen to be the best assay for both FGFR4 (90-125 kDa) and KLB (120kDa). Protein was isolated from the LNCaP progression model, HS27a bone marrow stromal cells and as a positive control, HepG2 hepatocarcinoma cells. FGFR4 and KLB have been studied extensively in HepG2 cells and these cells provide an excellent positive control cell for the following experiments. Mean intensity values for FGFR4 and β-actin band intensity were determined and used to calculate the FGFR4:β-actin ratio for analysis. In Figure 3.4, LNCaP, C4, C4-2, and C4-2B cells were each found to express approximately 4-fold higher FGFR4 protein when compared to HS27a cells, each with a p-value of <0.001 (Figure 3.4). These data provide further evidence that FGFR4 is more highly expressed in PCa cells and may play an important role in cellular signaling.

Similarly, PCa cells expressed approximately 4-fold higher KLB protein than compared to Hs27a cells, with a p-value of <0.001 (Figure 3.5). Taken together, these data suggest that FGFR4 and KLB are highly expressed in PCa cells when compared to BMSCs. Furthermore, the 4-fold higher expression of both FGFR4 and KLB in

PCa cells when compared to HS27a cells indicates the potential for formation of co-receptor pairs that would be required for potential FGF19-mediated metabolic signaling.

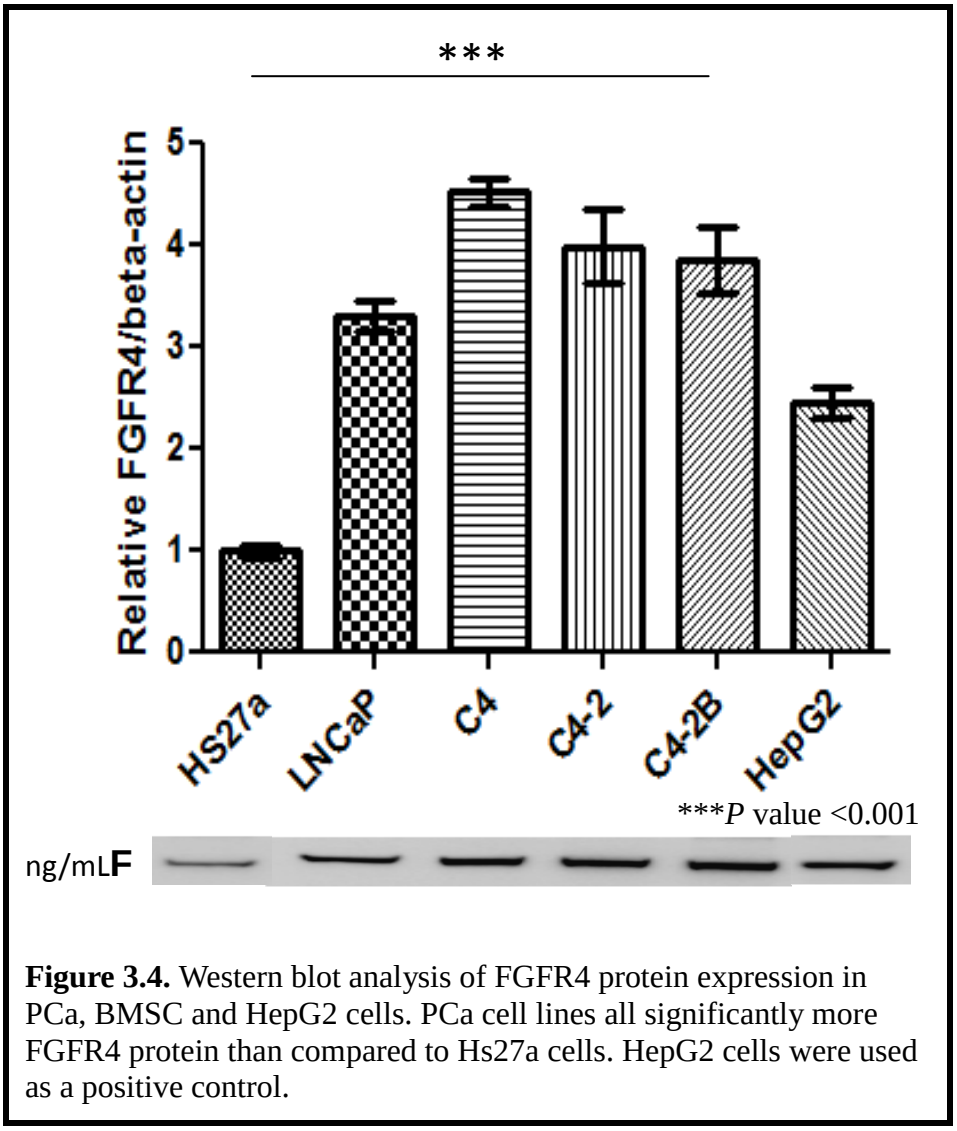
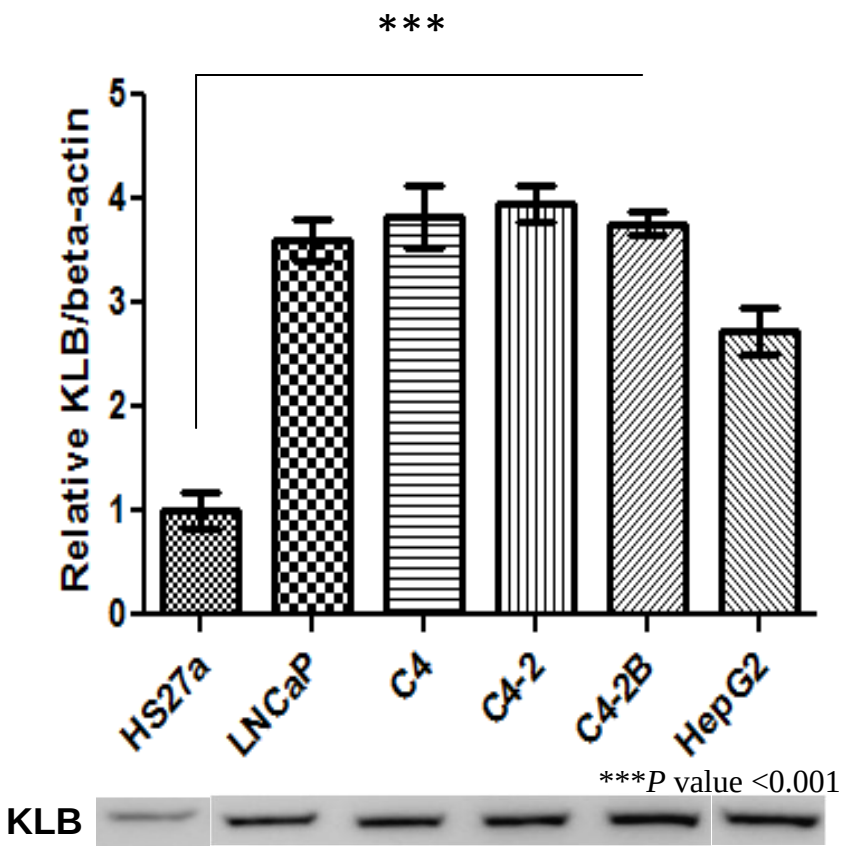


Figure 3.4. Western blot analysis of FGFR4 protein expression in PCa, BMSC and HepG2 cells. PCa cell lines all significantly more FGFR4 protein than compared to Hs27a cells. HepG2 cells were used as a positive control.



ERK**Figure 3.5.** Western blot analysis of KLB protein expression in PCa, BMSC and HepG2 cells. PCa cell lines all express significantly more KLB protein than compared to Hs27a cells. HepG2 cells were used as a positive control.

3.1.4. Immunofluorescence of FGFR4 and β-Klotho

To more fully identify differences of FGFR4 and KLB expression between PCa and bone stromal cells, I used immunofluorescence to stain C4, C4-2, C4-2B, and HS27a cells with mouse monoclonal antibodies to human FGFR4 and rabbit polyclonal antibodies to KLB, both of which were raised against an extracellular epitope of the respective receptor. HS27a cells were chosen for these experiments because they are structural stromal cells that do not secrete cytotoxic factors that would prohibit co-culture with PCa cells. The primary antibodies were detected by incubation with anti-mouse AlexaFlour 488 and anti-rabbit AlexaFlour 568. In Figure 3.6, C4-2B cells express strong, homogeneous signals for FGFR and KLB, both of which encompass the cytoplasm of each cell. C4-2 and C4 cells have more modest staining for both FGFR4 and KLB and when comparing the merged images, C4-2 and C4 cells have much more heterogeneous staining patterns of FGFR4 and KLB. HS27a bone stromal cells have little detectable FGFR4 and KLB when compared to C4-2B (Figure 3.6). As discussed previously, tumor cells are often surrounded by stromal cells and interactions between cancer cells and the surrounding microenvironment can drive various phenotypic alterations, such as FGF signaling. Similarly, bone metastatic PCa cells are surrounded by BMSC, and in order to visualize FGFR4 and KLB expression in a similar environment, I co-cultured C4-2B cells with HS27a BMSCs. In Figure 3.6, C4-2B cells expressed higher FGFR4 and KLB when compared to HS27a cells (Figure 3.6). Taken together, these data suggest that FGFR4 and KLB are more highly expressed and similarly distributed in PCa cells when compared to

bone stromal cells. Also, the expression of FGFR4 and KLB seemed to track the LNCaP progression model: higher levels of expression as the cell phenotype approaches a higher metastatic potential. Also, similar distribution of FGFR4 and KLB expression in C4-2B cells suggests possible co-localization required for effective FGF19 signaling.

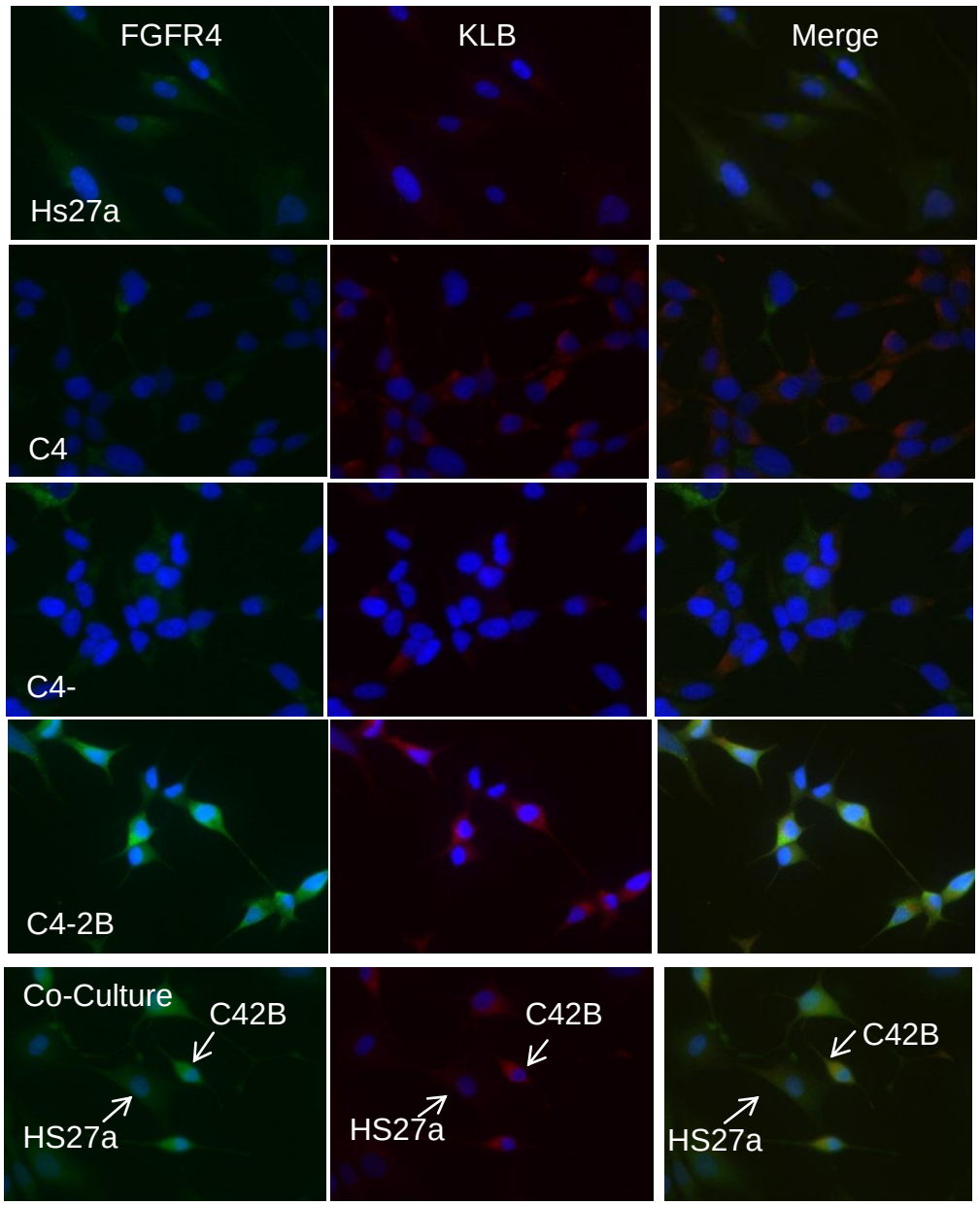


Figure 3.6. Immunofluorescent staining of FGFR4 and KLB in PCa and BMSCs. Imaged at 40X, HS27a cells have little FGFR4 (green) or KLB (red) signal. C4 and C4-2 cells have modest signal of both FGFR4 and KLB.C4-2B cells express strong, homogeneous signal for FGFR4 and KLB. In co-culture, HS27a and C4-2B cells were distinguished based on morphology the strong FGFR4 and KLB signals are clearly seen in C4-2B cells when compared to Hs27a.

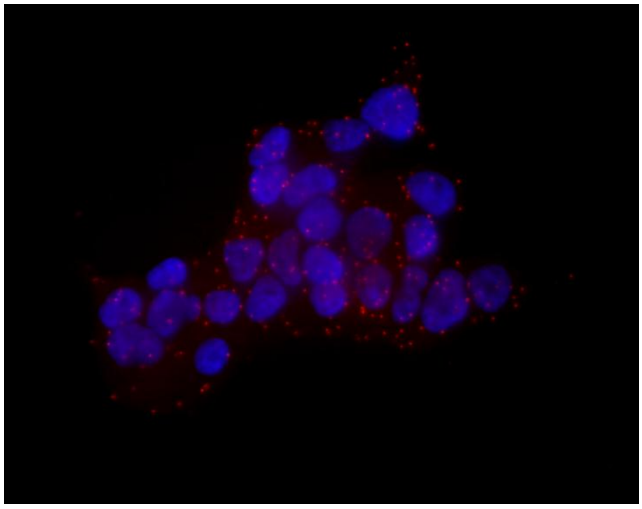
3.2. Association of FGFR4 and β-Klotho in PCa cells

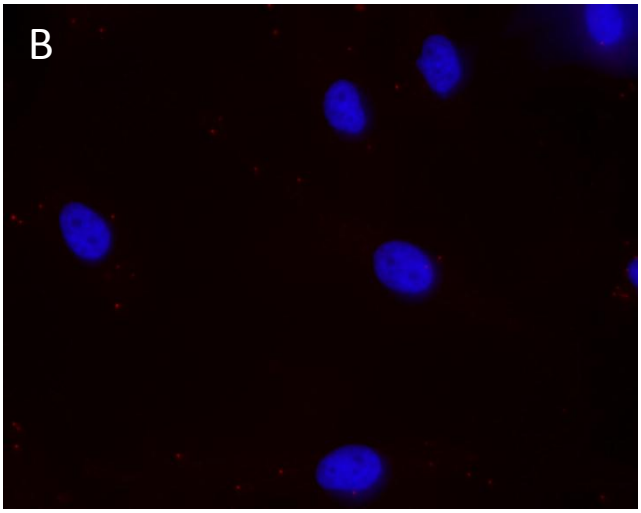
3.2.1. *In situ* co-localization of FGFR4 and KLB in C4-2B and HS27a cells.

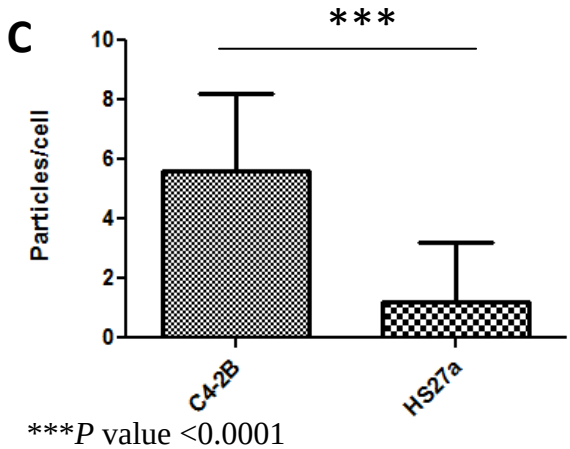
To determine co-localization of FGFR4 and KLB in PCa cells, I used a Duolink *in situ* Proximity Ligation Assay (PLA). This PCR amplification assay is capable of detecting protein interactions if the proteins of interest are within 40nm of each other. For the assay, I used the same mouse monoclonal antibodies to human FGFR4 and rabbit polyclonal antibodies to KLB that recognize extracellular epitopes of these two receptors discussed previously. C4-2B and HS27a cells were incubated with primary antibodies followed by anti-mouse and anti-rabbit secondary antibodies conjugated to unique DNA strands and linker oligonucleotides that allow the formation of a circularized DNA molecule. After incubation with a DNA ligase followed by DNA polymerase, that initiates rolling circle amplification, a concatamer of DNA is formed. Fluorescently-labeled complementary DNA probes hybridize to the concatamer and a high concentration of fluorescence can be detected for a FGFR4:KLB protein complex.

For this assay, three controls were analyzed in parallel to validate the specificity of various aspects of the assay: First, a technical positive control slide provided in the kit was used to determine the functionality of all PLA kit components reagents. A “no primary control” was run for each cell type by omitting either FGFR4 or KLB primary antibodies. Thirdly, a background control was used to both validate the specificity for FGFR4-KLB PLA signals by incubating the cells of interest with primary antibodies

targeting two proteins that are not expected to colocalize. FGFR4 and Neuron Specific Enolase (NSE) were used in this control and the resulting FGFR4-NSE PLA signal was subtracted from FGFR4-KLB PLA signals as nonspecific background signal. For image analysis, I followed a similar protocol as did Spears *et al*. by counting the number of PLA signals per cell after a background threshold was determined [40]. For quantification, FGFR4-NSE control PLA signals from each cell type were subtracted from the FGFR4-KLB PLA signals per cell and for statistical analysis, a Welch's corrected t test was used. As seen in Figure 3.7, approximately 5.6 FGFR4:KLB signals per cell were seen in C4-2B cells compared to 1.2 PLA signals per HS27a cell with a P values < 0.001(Figure 3.7). These data strongly suggest that FGFR4 and KLB form a complex in PCa cells and not in BMSCs.







***P value <0.0001

ERK**Figure 3.7.** *in situ* PLA of FGFR4 and KLB in C4-2B and HS27a cells. **A:** Representative image of C4-2B cells. PLA signal (Red); DAPI (Blue) **B:** Representative image HS27a cells. PLA signal (Red); DAPI (Blue) **C:** C4-2B cells express approximately 5.6 particles per cell compared to 1.2 particles per cell in HS27a. P value <0.0001

Chapter 4

Characterization of FGFR4 and β-Klotho signaling in PCa

4.1. FGF-mediated mitogenic, metabolic and survival signaling in PCa.

As discussed above, FGFRs are activated by distinct subsets of FGFs. Interestingly, FGF1 has been shown to activate all four FGFRs and although FGF1 is a universal activator, it does not elicit equal levels of activation across the FGFR family. FGF1 was shown to induce higher p-ERK1/2 phosphorylation in FGFR1 expressing cells than FGFR4 expressing cells [33, 35]. Conversely, FGF19 signaling in the setting of PCa bone metastasis has yet to be investigated. The vast majority of FGF19-FGFR4 studies have been performed in liver tissues, as FGF19 is primarily secreted into the ileum where it can be absorbed and pass into the blood stream. Locally, FGF19 elicits

strong metabolic changes in subcompartments of the liver [14, 42] and has the potential to initiate signaling in distal tissues that express FGFR4 and KLB.

4.1.1. ERK1/2 phosphorylation as a result of FGF1 but not FGF19.

To determine if the FGFR4-KLB complex activates the ERK/MAPK mitogenic pathway, I conducted a dose-response of C4-2B cells to FGF19. FGF19 was present in control patient serum at 205.3 $\pm$ 25.97 pg/mL [49]. Coordinately, PCa tumor cells may see similar concentrations of FGF19 and this was used as the lowest FGF19 treatment concentration. Other studies have used up to 300 ng/mL FGF19 to assay FGFR4-KLB function and this concentration was used as the maximal dose of FGF19 for the dose-response assay [21]. Accordingly, I treated C4-2B cells with 0, 2, 20, 50, 200 and 300 ng/mL of FGF19 followed by assaying ERK phosphorylation by western blotting. The same concentrations of FGF1 were used to compare ERK phosphorylation. FGF19 at 200ng/mL elicited maximal phosphorylation in FGF1 treated C4-2B cells compared to vehicle controls (Figure 4.1 (A)). Conversely, FGF19 did not elicit ERK phosphorylation at any concentration. To verify that the FGF19 was active, I treated HepG2 cells with 200 ng/mL FGF19 and assayed ERK phosphorylation (Figure 4.1 (B)). These data suggest that FGF19 does not activate the ERK/MAPK cascade and subsequent cell mitogenesis in C4-2B cells.

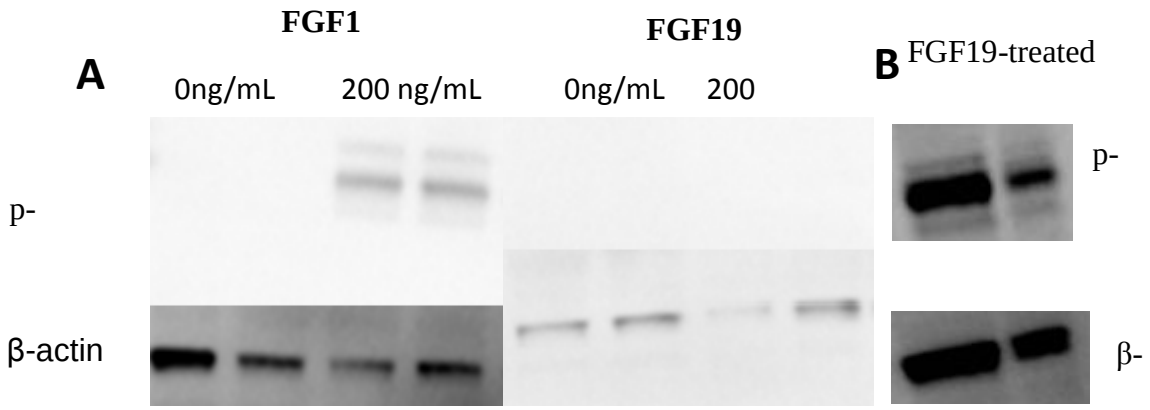


Figure 4.1. ERK1/2 phosphorylation in FGF1 and FGF19-treated C4-2B cells and FGF19-treated HepG2cells. FGF1-treated C4-2B cells show ERK phosphorylation at 42 and 44 kDa, respectively. FGF19 does not induce ERKphosphorylation in C4-2B cells but strongly activates ERK phosphorylation in HepG2 cells.

4.1.2. ACC2 expression in PCa bone marrow stromal cells

FGFR4-KLB signaling may be responsible for enhancing PCa metabolic signaling pathways for cellular growth and success in the bone marrow microenvironment, such as increase fatty acid oxidation and glucose uptake and metabolism [52]. To explore the role of potential FGFR4-KLB mediated metabolic signaling, I assayed acetyl coenzyme A carboxylase 2 (ACC2) mRNA expression in PCa and BMSCs. ACC2 is the rate limiting enzyme responsible for fatty acid biosynthesis. Specifically, ACC2 catalyzes the carboxylation of acetyl-CoA to malonyl-CoA which in turn inhibits the rate-limiting step in fatty acid uptake and oxidation by the mitochondria. In cells with high lipid oxidation rates, such as liver cells, reduced ACC2 expression would result in increased fatty acid uptake and oxidation, indicating an enhancement of cellular metabolism by way of fatty acid catalysis [6]. FGF19 treated mice have been shown

to have lower serum triglycerides and lower expression of ACC2 in the liver, suggesting higher fatty acid metabolism [6].

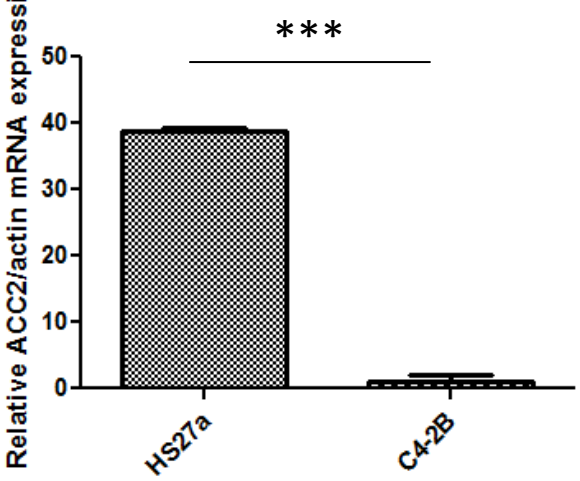


Figure 4.2. Relative ACC2 mRNA expression is approximately 39-fold higher in HS27a cells when compared to C4-2B cells. ****P* value <0.0001

To determine if PCa cells have enhanced cellular metabolism as a result of FGFR4-KLB signaling, I conducted a qRT-PCR of ACC2 mRNA in C4-2B and HS27a cells. C4-2B and HS27a cells grown under normal culture conditions described above. Upon analysis, untreated C4-2B cells express virtually no ACC2 mRNA as the qRT-PCR CT was near that of the corresponding "no template controls". Reciprocally, HS27a cells express approximately 39-fold higher ACC2 mRNA than C4-2B cells (Figure 4.2). These data suggest that C4-2B cells have the potential for high levels of fatty acid uptake and oxidation as a result of reduced ACC2 expression when compared to HS27a cells. Future studies should pursue this pathway further by investigating the fatty acid uptake differential between C4-2B and HS27a cells.

4.1.3. pS6 phosphorylation in PCa cells

To further characterize FGFR4-KLB regulation of cellular signaling, expression of phospho-S6 ribosomal protein (pS6) was assayed in the presence and absence of

FGF19 or FGF1. pS6 is mainly responsible for regulating the translation of mRNAs of proteins required for the cell cycle. To do so, p70 S6 kinase activates pS6 after the cell is stimulated by GFs. pS6 increases the translation of critical proteins and thus allows the cell cycle to progress. It may be beneficial to assay components of pS6, such as Phospho-4E-BP1 that is involved in the regulation of mRNA translation to understand this important pathway of cellular growth [45, 46].

To assay the role of FGFR4-KLB induction of pS6, C4-2B cells were treated with 200ng/mL FGF19 or FGF1 for 24 h. As seen in Figure 4.3., neither FGF19 nor FGF1 elicited an increase in baseline pS6. These data suggest that FGFR4-KLB does not increase pS6 and may not be involved with advancement of the cell cycle above that of canonical FGF signaling.

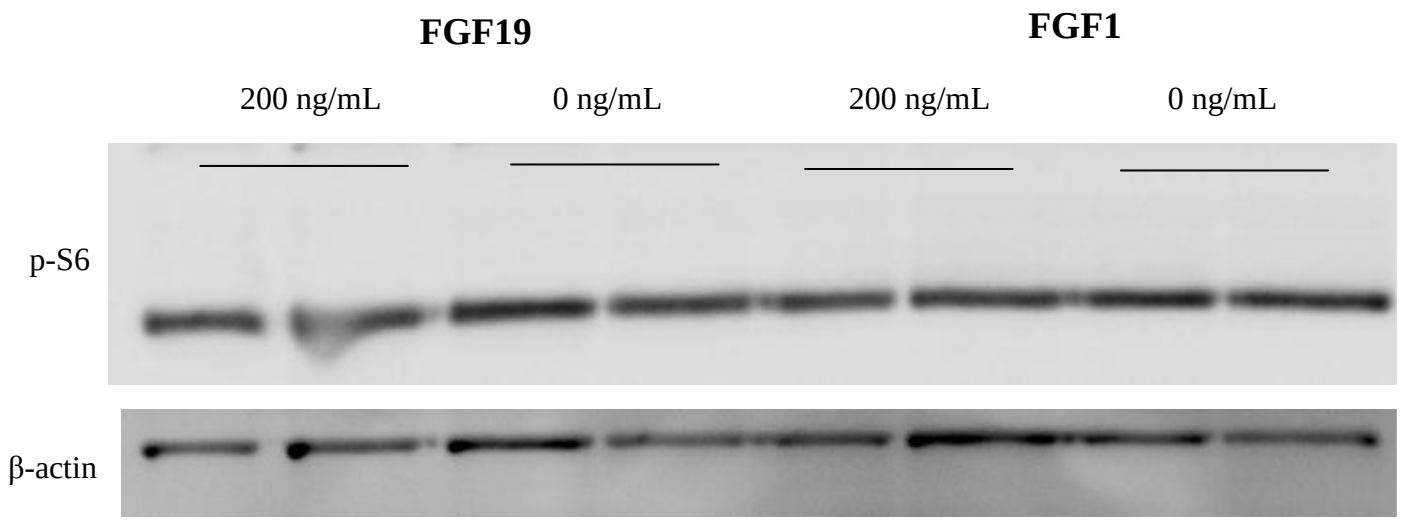


Figure 4.3. Expression of pS6 in C4-2B cells treated with FGF19 and FGF1 treatment. Neither FGF19 nor FGF1 treatment enhanced pS6 expression in C4-2B cells above untreated controls suggesting that FGF19 nor FGF1 are regulators of the pS6 cell-cycle regulation cascade.

4.2. FGF1 and FGF19-mediated resistance to chemotherapeutics

4.2.1. Camptothecin treatments

Camptothecin [CPT] is a long established anti-cancer compound known to induce apoptosis in PCa cells. This drug has been used extensively in studies involving C4-2B cells and provides an excellent avenue to explore the role of FGFR4-KLB in mediating resistance to chemotherapeutics [8].

To determine if FGFR4-KLB provides any resistance to chemotherapeutic-induced apoptosis, C4-2B cells were pretreated with FGF19 or FGF1 for 24h followed by 0.574 μM CPT. This concentration of CPT was determined to be the optimal concentration to inducing a sufficient apoptotic response in C4-2B cells grown on plastic by Gurski et al. [8]. Live/Dead staining was done following FGF and CPT treatments, providing qualitative data as to the degree of apoptotic resistance. As seen in Figure 4.4., C4-2B cells treated with vehicle appear morphologically ‘healthy’, as seen in the first column of bright field (BF)images, a majority of which have robust green fluorescent signal, indicating living cells and no detectible red fluorescent signal, indicating dead cells. Conversely, C4-2B cells treated with only CPT have a less healthy morphology, indicated by a rounder shape and more detached and floating cells. Although many of the CPT

treated cells have robust green fluorescence, far more of these cells have robust red staining, indicating cellular death. Additionally, the majority of the detached, floating masses of cells had little green fluorescence, a majority of which had high levels of red fluorescence (data not shown). Interestingly, cells pretreated with FGF19 and FGF1 also have robust green fluorescence; however, fewer dead cells are seen when compared to CPT only treatment. Morphologically, these cells did not appear to be as 'healthy' as vehicle treated cells as they appear more round and detached, but not to the extent seen in CPT only treatments, suggesting a potential FGF-mediated protection from chemotherapeutic-induced apoptosis.

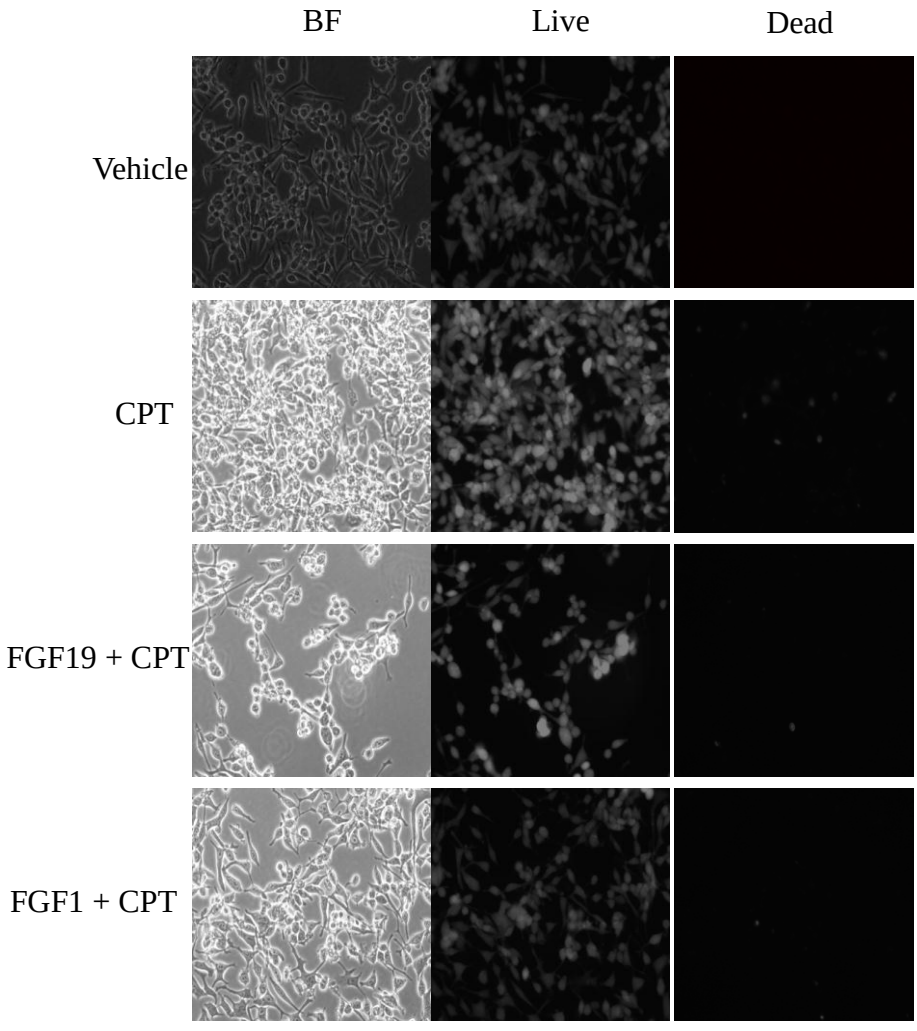


Figure 4.4.Live Dead assay of FGF19 and FGF1 suggesting pretreatment may protect PCa cells from chemotherapeutics. Cells were pre-treated with FGF19 or FGF1 and visualized at 40X. Cells pretreated with 200ng/mL FGF1 or 200ng/mL FGF19 look healthier (BF) and have fewer dead cells when compared to vehicle controls (Dead).

4.3. β-Klotho knockdown

To help elucidate biological significance of FGFR4 and KLB expression in PCa cells, I conducted siRNA experiments to reduce KLB mRNA. KLB siRNA was chosen over FGFR4 siRNA due to the requirement of KLB for all FGF19 mediated signaling with FGFR4. If FGFR4 siRNA were used, all FGFR4 signaling, both canonical and KLB-mediated would be disrupted, rather than the KLB-FGFR4 pathway specifically. Three KLB siRNAs were purchased from Origene and prepared as per manufacturer's protocol. After 24 h incubation with the siRNA, cells were treated with FGF19 or FGF1. Subsequently, protein was isolated and western blots were probed for KLB, FGFR4, pS6 and β-actin. As seen in Figure 4.5, none of the three different KLB-targeted siRNAs were able to effectively knock down KLB transcripts below levels observed with scrambled siRNA control. The untreated control KLB band is much stronger than any of the siRNA treatments and this can be attributed to a loss of cells after 24 h incubation with the respective FGFs. These cells were in the presence of the transfection reagent for a longer period of time which would account for the lower overall protein expression (Figure 4.5).

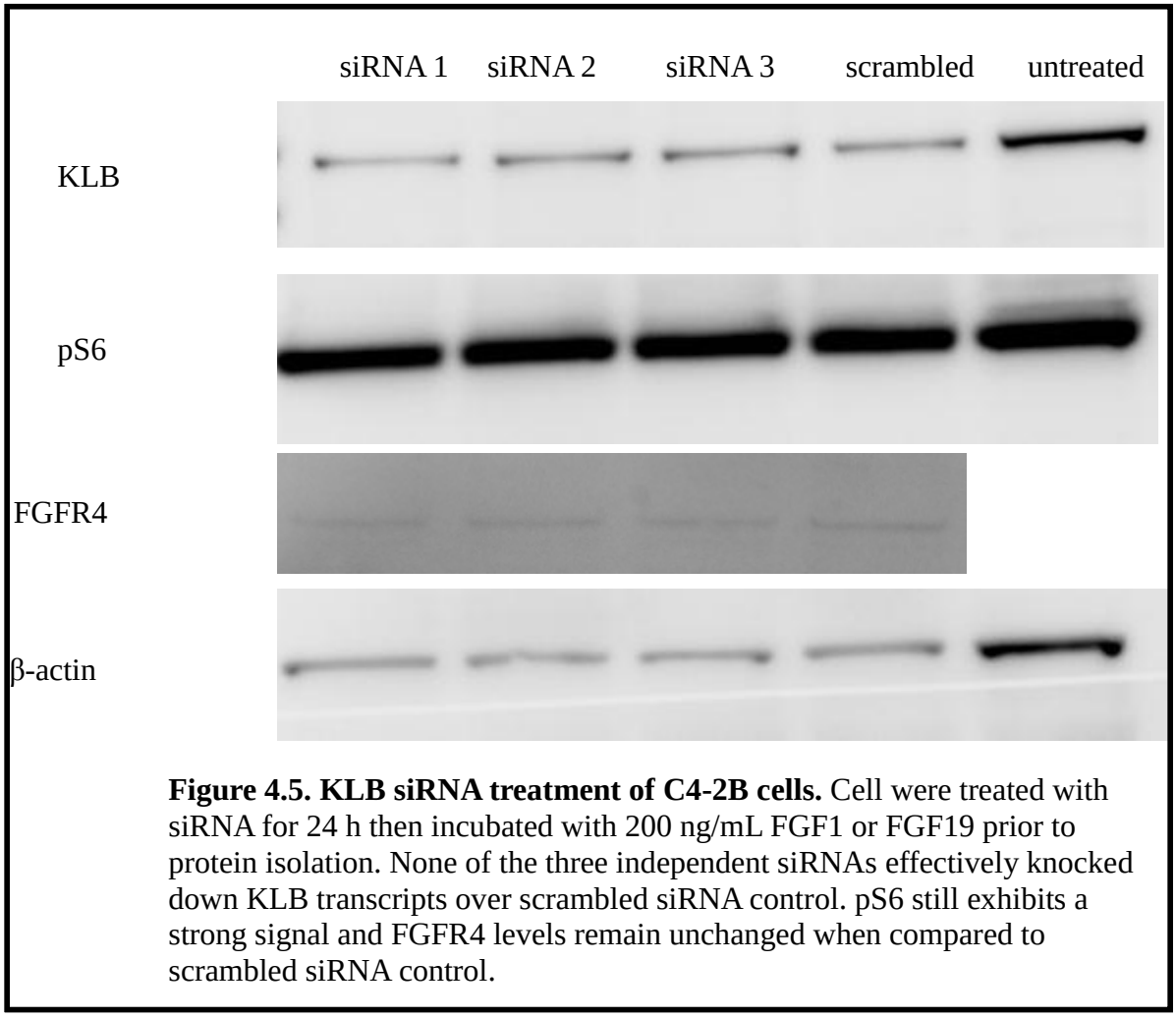


Figure 4.5. KLB siRNA treatment of C4-2B cells. Cell were treated with siRNA for 24 h then incubated with 200 ng/mL FGF1 or FGF19 prior to protein isolation. None of the three independent siRNAs effectively knocked down KLB transcripts over scrambled siRNA control. pS6 still exhibits a strong signal and FGFR4 levels remain unchanged when compared to scrambled siRNA control.

Chapter 5

Discussion

5.1. General Discussion

The rate of PCa progression between individual patients can be very difficult to predict. For some, it is very slow growing and can go largely unnoticed. For others, PCa can progress very rapidly. When PCa cells metastasize to bone, the disease is often very painful and ultimately lethal. The bone microenvironment is quite inhospitable to normal prostatic epithelial cells that retain many epithelial properties [38]. In order for metastatic PCa cells to adapt and survive in the hostile bone microenvironment, they must adopt bone-like phenotypes by upregulating osteoblastic signaling molecules. FGF signaling pathways also have been linked to initiation, progression and aggressiveness of PCa [16, 21]. In clinical disease, overexpression of FGFs and coordinate FGFRs, are considered hallmarks of metastatic cancer cell adaptation that promote increased cellular motility and tumor invasiveness, angiogenesis, and inhibition of apoptosis that

increases cancer cell survival [51]. Cancer cells often rely upon interactions and signaling from surrounding stromal cells to obtain phenotypic changes that enable PCa cell survival [50].

The FGFR family is comprised of four distinct receptors, FGFRs-1-4, with FGFR-4 being most closely-associated with PCa progression in many cancer cells. FGFR4 is activated by the FGF1, FGF8, FGF9, and FGF19 subfamilies, which, upon binding HSPG-receptors such as syndecan-1 or perlecan, bind to FGFR4, causing dimerization and transphosphorylation. Interestingly, the FGF19 weakly binds HSPGs, allowing FGF19 to diffuse more freely through the extracellular matrix and act as an endocrine signaling factor. However, the reduced heparin-binding affinity also reduces the ability of FGF19s to bind FGFRs. In order for FGF19 and FGFR4 to interact the KLB coreceptor is required. The Klotho gene family is associated with regulating lipid and glucose metabolism in the liver through interactions with FGFRs. KLB is primarily expressed in the liver and, to lesser extent, in the prostate and kidneys. KLB expression is associated with inhibition of apoptosis and stimulation of angiogenesis, however, the role of KLB and its interplay with FGF signaling in PCa remains unclear.

Because of the importance of FGF signaling in PCa progression and the unknown nature of KLB in PCa, this study focused on the investigation of these two important signaling molecules throughout disease progression.

5.2. FGFR4 and KLB are highly expressed in PCa cells.

To first explore the role of FGFR4 and KLB in PCa, I assayed mRNA levels for these proteins in both PCa and BMSCs. As discussed above, FGFR4 has been associated with PCa aggressiveness and progression [30]. If PCa cells express higher levels of FGFR4 than surrounding BMSCs, the cancer cells can exploit an arm of FGF signaling that remains inaccessible to the surrounding stroma. Previous work in our lab found high FGFR4 mRNA expression in C4-2B cells when compared to HS27a cells [23]. To obtain more comparative FGFR4 mRNA expression, I conducted qRT-PCR experiments and found high FGFR4 expression in all members of the LNCaP progression model of PCa when compared to HS27a cells (Figure 3.1). Interestingly, FGFR4 expression was highest in C4-2 and C4-2B cells, those associated with metastasis and specifically osteoblastic bony metastasis, respectively (Figure 3.1). These data suggest that PCa cells express higher levels of FGFR4 especially in later stages of disease. Higher expression of FGFR4 in the latter stages of disease may provide PCa cells that have metastasized to some of the most hostile tissues, like bone, to exploit signaling cascades by way of altered receptor expression profiles that would otherwise be unavailable to normal prostatic epithelial cells in the same compartment. This altered receptor expression and signaling patterns may allow these adaptive PCa cells to survive and thrive, leading to larger metastases as well as further spread of the disease.

Similar to the FGFR4 experiments discussed above, I first conducted KLB RT-PCR experiments to determine the presence of KLB transcripts in C4-2B and HS27a cells. Again, C4-2B cells expressed a strong signal for KLB when compared to HS27a cells (Figure 3.2). Again, it was important to obtain comparative KLB mRNA expression between PCa and BMSCs. By way of qRT-PCR, all members of the LNCaP expression model of PCa express high levels of KLB mRNA when compared to HS27a cells (Figure 3.3). Interestingly, KLB expression levels reach a maximum earlier that what was seen with FGFR4 transcripts. LNCaP cells express approximately 16-fold higher KLB mRNA than HS27a cells while C4, C4-2 and C4-2B cells express approximately 24-fold higher KLB mRNA (Figure 3.3). These data not only demonstrate that KLB mRNA is expressed at higher levels in PCa cell lines, but also suggest that KLB expression also reach a maximum sooner in disease progression, when cells begin to form tumors (Figure 1.1). The early peak of KLB in PCa progression may be used as a marker to distinguish true PCa from other conditions of the prostate like benign prostatic hyperplasia that may share similar markers like PSA.

FGF signaling relies upon cell-surface protein expression to bind the appropriate FGFs and transmit intracellular signaling cascades. To determine FGFR4 and KLB protein expression in PCa and BMSC models, I conducted western blot experiments. Similarly to FGFR4 mRNA expression, FGFR4 protein is approximately 4-fold higher in C4-2B cells when compared to HS27a cells (Figure 3.4). FGFR4 protein expression also peaks in the LNCaP progression model when PCa cells are metastatic and tumorigenic as seen in FGFR4 mRNA expression. These data suggest that FGFR4 expression occurs early in metastasis, potentially providing enhanced FGF signaling and survival of PCa cells in hostile environments.

KLB protein is also highly expressed in PCa cells when compared to BMSCs (Figure 3.5). Consistent with KLB mRNA expression, KLB protein expression peaks early in PCa progression, when cells are tumorigenic and non-metastatic. These data suggest that KLB protein is present in PCa cells and along with FGFR4 expression, may provide FGF signaling avenues unavailable to the surrounding stroma thus allowing PCa cells to survive and thrive in an otherwise hostile microenvironment.

It has been shown that the formation of the FGFR-KLB complex is required for many signaling cascades, both mitogenic and metabolic [6]. In an initial attempt to both visualize FGFR4 and KLB protein expression and proximity of these two receptors, I immunofluorescently stained PCa and BMSCs. FGFR4 and KLB fluorescent signals are highest in C4-2B cells where a coordinate similar distribution of FGFR4 and KLB is seen (Figure 3.6). When C4-2B and HS27a cells were co-cultured, a stark distinction of FGFR4 and KLB can be seen in C4-2B cells, thus providing a glimpse into the expression levels and similar distribution of this co-receptor pair in an environment similar to what may be seen in bone metastatic PCa. These data provide further evidence that FGFR4 and KLB are highly expressed and similarly distributed in PCa cell lines when compared to BMSC lines. As with altered FGFR4 expression and the potential enhancement of FGFR4 signaling, the addition of high KLB expression may provide PCa cells access to signaling molecules and cascades normally restricted to the liver and other gastrointestinal organs. In doing so, the ability to utilize endocrine-like FGF19 for signaling by PCa cells in the bone would leave PCa cells expressing high FGFR4-KLB as the sole consumers of this FGF19 in the bone, providing a unique avenue for enhanced survival and another signaling pathway to exploit for enhanced survival in the bone microenvironemnt.

5.3. Association of FGFR4 and KLB

Although the aforementioned immunofluorecence data provided insights into the similar distribution of FGFR4 and KLB, it was still unclear whether these co-receptors, in fact, associate, thus providing the potential for enhanced cellular signaling. To determine if FGFR4 and KLB associate and form a co-receptor pair, I conducted an *in situ* Proximity Ligation Assay (PLA) targeting FGFR4 and KLB. If FGFR4 and KLB are within 40 nm of each other, a strong red fluorescent signal will result. More FGFR4-KLB complexes were found in C4-2B cells compared to HS27a cells (Figure3.7 A and B). These data suggest that FGFR4 and KLB are physically very close therefore, may form functional co-receptor pairs in PCa cells. Being that FGFR4 and KLB are both highly expressed and seem to be associated, forming co-receptor pairs in PCa cells is very promising for investigating the signaling potential of these two molecules. With a functional coreceptor pair, PCa cells have the potential access to signaling molecules and cascades normally restricted to cells of gastrointestinal organs, providing a unique avenue for enhanced survival signaling.

5.4. FGFR4-KLB-mediated mitogenic and metabolic signaling in PCa

Many FGFs are able to elicit signaling responses upon binding to FGFRs, including FGF1 and other ‘canonical’ FGFs. FGF19 is unique in that it requires the presence of

FGFR4 and KLB in order to effectively alter downstream signaling pathways. To this end, I treated PCa cells with FGF1 and FGF19 to determine the levels of both mitogenic and metabolic signaling.

The ERK/MAPK cascade is a well-studied mitogenic pathway and accordingly, I assayed ERK phosphorylation in PCa cells in response to FGF1 and FGF19 treatments. In Figure 4.1, FGF1 was able to elicit expression of pERK1/2 suggesting that the FGF-FGFR signaling pathways are intact in PCa cells. However, FGF19 treatments were unable to elicit such a response (Figure 4.1). These data suggest that although FGFR4 and KLB are highly expressed and associated in PCa, they do not regulate ERK/MAPK mitogenic pathways in PCa. These findings are not completely surprising in that FGFR4 has been shown to only weakly activate pERK1/2 when compared to FGFR1 so FGFR4-KLB may play a larger role in other signaling pathways both mitogenic and metabolic [33, 35].

FGF19 is a well-studied metabolic regulator in liver tissue and other organs of the gastrointestinal tract [6]. FGF19 has been shown circulating in the blood plasma of control patients, suggesting that PCa patients would have circulating FGF19 that PCa cells would be able to utilize for signaling due to high expression of FGFR4 and KLB. In the same study, mice treated with FGF19 had low express levels of ACC2, suggesting higher fatty acid metabolism and increased cellular metabolism.

Accordingly, I first assayed ACC2 expression in untreated C4-2B and HS27a cells. C4-2B cells expressed virtually no ACC2 when compared to HS27a cells suggesting these cells have already enhanced their ACC2-mediated cellular metabolism. Although these data proved to be a dead-end for further studies involving FGF19 treatments and KLB knockdowns, it provided important insights into the cellular metabolism of PCa cells. This is not to say that FGFR4-KLB does not regulate PCa cellular metabolism, rather, FGFR4-KLB may be involved in other arms of cellular energetics that the present study did not address.

To further explore FGFR4-KLB signaling in PCa, I assayed the expression of a potent regulator of the expression of proteins critical for regulation of the cell cycle in response to FGFs, pS6. Upon analysis, neither the presence of FGF1 or FGF19 altered the expression of pS6 in PCa cells above baseline no treatment controls (Figure 4.3). This suggests that FGFR4-KLB is not a regulator of pS6. I cannot completely rule out a role of FGFR4-KLB in regulation of other cell cycle regulating components, thus further work will be required to determine if any other aspects of the cell cycle are altered by this complex.

5.5. FGFR4-KLB may provide resistance to chemotherapeutics

Although the data presented above is promising for the signaling potential of FGFR4-KLB in PCa, a biologically relevant experiment is needed to illustrate the functionality of KLB-FGFR4. Accordingly, I treated C4-2B cells with a well-studied anti-cancer compound, camptothecin (CPT). In our lab, CPT has been extensively studied with C4-2B cells and I was able to use these as a guide for CPT doses [8]. Many C4-2B cells treated with CPT alone were shown to havehigh levels of apoptosis when compared to vehicle controls (Figure 4.4). Interestingly, cells pretreated with FGF1 and FGF19 exhibited fewer dead cells and live cells with a similar morphology to vehicle controls. These data suggest that both FGFR4-KLB activity and FGF1 pre-treatment may provide some degree of protection from chemotherapeutics. However, many of the cells in the FGF19 and FGF1 treatment groups were lost during various washes for removal of the CPT solution and could not be imaged so further data will be required to conclusively determine if FGFR4-KLB may be providing protection against chemotherapeutic agents. Most commonly, cancer cells upregulate drug transporter proteins or alter DNA repair mechanisms to achieve chemoresistance and this may also be the case in PCa [53].

5.6. siRNA knockdown of KLB

I conducted siRNA knockdown experiments targeting KLB in order to more completely determine the biological significance of high FGFR4-KLB expression and functionality in PCa cells. Unfortunately, none of the three independent KLB siRNAs were able to effectively knock-down KLB protein below the scrambled siRNA control (Figure 4.5). Not surprisingly, pS6 and FGFR4 protein expression levels remained unchanged in all treatment pools. KLB siRNAs may not be providing adequate knockdown for a variety of reasons. A recent study by Amarzguioui *et al.* found that longer siRNAs of 25nt as opposed to the traditional 21 nt siRNAs are able to "serve as dicer substrates" thus providing better interference [48]. Additionally, the siRNAs used in this study may need to be incubated with the cells for a longer period of time than 24 h. The half-life of KLB protein may be longer than anticipated so only incubating the cells with KLB siRNA for 24 h may not provide effective protein-level knockdown. Accordingly, future work should include longer KLB siRNA treatments (48 or 72 h).

5.7. Future Work

This study has conclusively shown high expression and association of FGFR4 and KLB in the LNCaP progression model of PCa compared to a BMSC model, HS27a. However, much work is required to investigate the role of FGFR4-KLB signaling in the setting of PCa.

The association of FGFR4 and KLB as assayed with PLA is evident in C4-2B cells. It would be beneficial to conduct this assay in the other LNCaP cell lines to determine when along PCa progression FGFR4 and KLB are able to form a complex. Additionally, clinical samples of PCa should be examined for the association of FGFR4 and KLB. As discussed above, FGFR4 and KLB protein expression reach a maximum of expression at different stages of progression and coordinately, they may only form co-receptor pairs at certain stages of disease progression.

Although FGFR4-KLB did not induce ERK phosphorylation, this co-receptor pair may be involved in other mitogenic and cell-survival pathways. It would be beneficial to assay the state of the AKT cell survival pathway in the presence of FGF19.

For a firm understanding of FGFR4-KLB signaling in PCa, siRNA knockdown experiments will be key. As discussed above, I was unable to obtain effective KLB siRNA knockdown. If effective knockdown is attained, assaying pS6 and FGFR4 expression should be done. If KLB is knocked down and pS6 levels are also reduced in the presence of FGF19, FGFR4-KLB may be playing a role in regulating the cell cycle. As was shown in Figure 4.3, FGF19 treatments did not induce further pS6 expression; however, in this state, the PCa cells may already be expressing maximal pS6 so treatment with FGF19 would have no effect and it would appear that FGFR4-

KLB does not play a role in cell-cycle regulation. If however, FGFR4-KLB is involved in cell cycle regulation and KLB was effectively knocked down, we may see reduced pS6 expression that could not be rescued with FGF19 administration. This would place FGFR4-KLB as a regulator of the cell cycle.

FGFR4-KLB may provide PCa cells with protection from chemotherapeutics. However, more data is required to determine in this chemotherapeutic protection is the case. In my experiment, cells were first pre-treated with FGFs for 24h, FGFs were removed, and CPT was incubated with the cells for an additional 24h. After CPT treatment, multiple washes were done to remove the CPT and in the process, many cells were lost as is common with C4-2B cells. Additional care during these washes is required to prevent cellular loss to obtain adequate sample sizes for analysis. It may also be beneficial to obtain quantitative data of live/dead cells for a more complete study.

5.8. Summary and Significance of Findings

In summary, I determined that FGFR4 and KLB mRNA and protein are highly expressed in the LNCaP progression model of PCa when compared to the HS27a cell line model of BMSCs. I was also able to use PLA to show that FGFR4 and KLB are within 40 nm of each other forming a co-receptor pair. Surprisingly, FGFR4-KLB did not regulate ERK phosphorylation or pS6 expression. However, ACC2 expression was greatly reduced in C4-2B cells and FGFR4-KLB may provide chemotherapeutic resistance to PCa cells. As discussed above, FGFR4 has been

associated with PCa aggressiveness while KLB has only been associated with metabolic signaling associated with the liver. This study has shown expression of both receptors as well as a high degree of colocalization on PCa. The combinatorial expression of both receptors and formation of a functional co-receptor pair may allow PCa cells to utilize previously unknown signaling pathways enhancing PCa survival and success in the bone. FGFR4-KLB expression and signaling pathways may provide excellent targets for future therapies.

5.9. Final Thoughts

Although FGFR4-KLB have yet to be demonstrated to be regulators of cellular signaling, the presence of these proteins at high levels in PCa models make them attractive targets for future therapies. Recently, the Farach-Carson laboratories in conjunction with Dr. Daniel Wagner, Dr. Dmitri Lapotko and Dr. Kate Hleb (Rice University) have begun to investigate the effectiveness of plasmonic nanobubble-mediated therapies. In early work to develop an *in vivo* model for PNB-mediated PCa cell detection and ablation, the Wagner lab xenografted C4-2B cells into zebrafish embryos and observed C4-2B cell localization in ventral tail fin of these embryos [34]. With a reliable PCa xenograft model in place, targeting multiple highly expressed cell surface receptors with antibody-mediated gold nanoparticles is highly desirable because tumors and populations of cancer cells residing in the tumor are quite heterogeneous. In their previous work, it was shown that combined. Prostate Specific Membrane Antigen (PSMA) and Epidermal Growth Factor Receptor (EGFR) targeted nanoparticles for plasmonic nanobubble PCa cell ablation *in vitro* has been quite effective [18, 20, 34, 48]. FGFR4-KLB may be an even better target to increase selectivity and effectiveness of this technology as these proteins form

coreceptor pairs, unlike PSMA and EGFR. Having a coreceptor pair may more easily facilitate nanoparticle cluster formation and nanobubble generation due to two reasons: first, the close proximity of the two receptors allow nanoparticles bound to each receptor may be up taken into the cell in the same endocytic vesicle, as opposed to different vesicles that result from distant receptors. Receptors in close proximity would more readily form vesicles with many nanoparticles, making nanobubble formation easier and more specific to cancer cells. Second, by selecting two receptors specifically expressed at high levels in cancerous tissue would allow for greater therapeutic specificity, thus reducing damage to surrounding non-cancerous tissues.

By furthering our understanding of FGFR4-KLB signaling and targeting FGFR4-KLB for therapeutics, new avenues of novel cancer treatments may come to light.

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